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(54) Title: IMMUNOMODULATORY FRAGMENTS OF POLYCLONAL ANTILYMPHOCYTE GLOBULINS (ALGs) AND USES THEREOF

(57) Abstract

This invention concerns novel $F(ab')_2$ and Fab fragments of polyclonal antilymphocyte globulins (ALGs) that surprisingly have immunomodulatory activity, wherein those immunoglobulin fragments may be obtained, for example, from polyclonal antithymocyte globulin (ATG) preparations comprising antibodies directed against lymphocyte cell surface proteins. Contrary to the well known immunosuppressive activity of intact ALG/ATG preparations, $F(ab')_2$ and Fab fragments derived therefrom retain their immunomodulatory activity without the accompanying massive and long–lasting lymphocytopenia associated with the administration of intact ALG or ATG preparations. Moreover, the herein described $F(ab')_2$ and Fab fragments retain immunomodulatory activity without retaining F_c receptor binding activities or the ability to bind to complement. When combined with lymphocyte cells either *in vivo*, *ex vivo* or *in vitro*, these $F(ab')_2$ and Fab fragments have immune system modulating activity and may be employed in a variety of different applications.

IMMUNOMODULATORY FRAGMENTS OF POLYCLONAL ANTILYMPHOCYTE GLOBULINS (ALGs) AND USES THEREOF

FIELD OF THE INVENTION

The field of this invention is fragments of polyclonal antilymphocyte globulins (ALGs) for immunomodulatory purposes.

BACKGROUND OF THE INVENTION

Antilymphocyte globulins (referred to herein as ALGs) and the subset antithymocyte globulins (referred to as ATGs) are powerful biological immunosuppressive agents that are currently used in clinical organ transplantation for prophylaxis and treatment of acute rejection crises, in allogeneic bone marrow transplantation for the prevention and treatment of acute graft-versus host disease and in the treatment of severe bone marrow aplasia (Bonnefoy-Bérard et al., *Blood* 83:1051-1059 (1994) and Bourdage and Hamlin, *Transplantation* 59:1194-1200 (1995)). Polyclonal ALGs and ATGs are often obtained by immunizing rabbits or horses with human lymphocytes or human T cells, respectively, to provide ALG and ATG preparations containing IgG antibodies directed against a wide range of different lymphocyte or T cell surface antigens, some of which are expressed exclusively by lymphocytes while others are shared by different cell lineages (Bonnefoy-Bérard et al. (1994), *supra*, Bourdage and Hamlin (1995), *supra*, Rebellato et al., *Transplantation* 57:685-694 (1994), Bonnefoy-Bérard et al.,

Transplantation 51:669-673 (1991) and Raefsky et al., *Blood* 68:712-719 (1986)). Examples of lymphocyte cell surface antigens against which ALGs or ATGs may be directed include CD1, CD2, CD3, CD4, CD5, CD6, CD7, CD8, CD11, CD16, CD18, CD20, CD28, CD38, CD40, CD44, CD45, CD56, CD58, MHC class I, MHC class II and the T cell receptor.

While the administration of ALGs/ATGs has proven to be useful for the prevention and treatment of acute transplantation rejection crises, acute graft-versus host disease and bone marrow aplasia, it also exhibits some significant adverse physiological effects. For example, ALG/ATG administration induces a massive and long-lasting lymphocytopenia that entails the risk of a general overimmunosuppression with an increased incidence of severe and/or prolonged viral infections and virus-associated tumors (e.g. herpes virus and papillomavirus). In addition, ALG- or ATG-induced lymphocyte deletion also results in the elimination of suppressor T cells that are necessary for the induction of graft acceptance or downregulation of self-reactive T cells causing autoimmune disease. It is evident, therefore, that immunosuppressive treatment with ALGs and/or ATGs, however effective, also has some significant disadvantages.

Given the above, medical researchers have recognized a need to provide novel methods for modulating immune responses without the adverse side effects associated with the administration of polyclonal ALGs/ATGs. In response to this need, we herein demonstrate and describe for the first time that the administration of $F(ab')_2$ and/or Fab fragments of polyclonal ALGs or polyclonal ATGs avoids the induction of the massive lymphocyte depletion and non-specific immunosuppression that is observed during treatment with intact ALGs/ATGs while remarkably retaining immunomodulatory activity.

SUMMARY OF THE INVENTION

The biological properties of ALGs and ATGs have been extensively investigated but it is only recently that major functional properties of the $F(ab')_2$ or Fab antigen binding fragments of ALGs and ATGs could be delineated and shown herein to be independent from the complement binding and Fc receptor binding activities that are associated with the Fc portion of IgG molecules. Given the present disclosure, it is now possible to prepare $F(ab')_2$ and/or Fab fragments of ALGs/ATGs which surprisingly retain some of the immunomodulatory activities of intact ALGs/ATGs while avoiding the induction of massive lymphocyte depletion and non-specific immunosuppression observed during ALG or ATG treatment. In addition, the use of ALG and/or ATG $F(ab')_2$ and/or Fab fragments allows the induction of graft acceptance and the reversal of autoimmune and graft versus host disease.

In accordance with one embodiment of the present invention, we herein provide a method for modulating a lymphocyte-mediated immune response which comprises contacting lymphocytes with an immunomodulating amount of $F(ab')_2$ and/or Fab fragments derived from a polyclonal preparation of anti-lymphocyte immunoglobulins, wherein the immune response is modulated. In a particularly preferred embodiment, the polyclonal antilymphocyte globulin (ALG) preparation from which the fragments are derived is a polyclonal antithymocyte globulin (ATG) preparation. The polyclonal ALG or ATG preparations may be obtained by immunizing a non-human animal such as rabbit or horse with human lymphocytes or T cells, respectively, or may be obtained by immunizing a non-human animal with a cell line engineered to recombinantly express one or more human lymphocyte-associated cell surface antigen(s).

Another embodiment of the present invention is directed to a method for suppressing an immune response in a mammal which

comprises administering to the mammal an immunosuppressing amount of $F(ab')_2$ and/or Fab fragments of polyclonal ALGs and/or ATGs, wherein the immune response is suppressed. The immune response that is suppressed by the administration of the $F(ab')_2$ and/or Fab fragments may be associated with one or more disease conditions or disorders including, for example, tissue graft rejection, autoimmune diseases such as rheumatoid arthritis, insulin-dependent diabetes mellitus (IDDM), colitis, Crohn's disease, multiple sclerosis, and the like, Alzheimer's disease, asthma, etc. For the prevention or treatment of graft rejection, $F(ab')_2$ and/or Fab fragments may be administered before and/or after the tissue graft is introduced into the host, wherein the tissue graft may be an intact organ or cells.

Yet another embodiment of the present invention is directed to a method for activating a lymphocyte-mediated immune response which comprises contacting lymphocytes with an immunoactivating or mitogenic amount of $F(ab')_2$ fragments of anti-lymphocyte immunoglobulins, wherein the anti-lymphocyte immunoglobulins fragments may be derived from a polyclonal ATG preparation.

An additional embodiment of the present invention is directed to a composition of matter comprising an antilymphocyte antibody that does not substantially bind to either complement or F_c receptors, wherein the antibody has immunomodulatory activity. In a preferred embodiment, the antibody binds to a protein on the surface of T lymphocytes.

Additional embodiments of the present invention will become evident to the skilled artisan upon a reading of the present specification.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. ATG $F(ab')_2$ activate human PBLs .

Figure 1 shows activation of PBMCs and PBLs in medium alone (med), or in the presence of ATGs, $F(ab')_2$ fragments of ATGs, the antibody OKT3, PHA or PMA plus ionomycin (P/I).

Figure 2. ATG and $F(ab')_2$ fragments induce incomplete T cell activation at submitogenic concentrations. Figure 2 shows the ability of ATGs and $F(ab')_2$ fragments thereof to induce T cell activation as measured by the expression of the T cell markers CD69, Fas and CD25.

Figure 3. ATGs and $F(ab')_2$ fragments thereof down-regulate surface expression of T cell functional molecules. Figure 3 shows the ability of ATGs or $F(ab')_2$ fragments thereof to down-regulate the expression of various T cell-associated cell surface markers including CD3, CD4, CD2, CD8, CD11a and the T cell receptor (TCR).

Figure 4. Modulation of T cell surface molecules induced by ATGs or $F(ab')_2$ fragments thereof is associated with hyporesponsiveness to stimulation with anti-CD3 antibody. Figure 4 shows the ability of ATGs or $F(Ab')_2$ fragments thereof to alter the ability of T cells to respond to anti-CD3 stimulation.

Figure 5. ATGs or $F(ab')_2$ fragments thereof inhibit the proliferation of alloreactive T cells. Figure 5 shows the ability of ATGs or $F(ab')_2$ fragments thereof to inhibit the proliferation of T cells in the presence of allogenic stimulator cells.

Figure 6. ATGs and $F(ab')_2$ fragments thereof induce apoptosis of activated but not resting T cells. Figure 6 shows the ability of ATGs, $F(ab')_2$ fragments thereof, normal rabbit immunoglobulin (Ig)

and anti-CD95 antibody (CH11) to induce apoptosis of activated or resting T cells.

Figure 7. Inhibition of T cell proliferation by Fab fragments of ATGs. Figure 7 shows the ability of Fab fragments of ATGs to inhibit cell proliferation as compared to a control Fab fragment.

Figure 8. ATG fragments do not bind to human complement. Figure 8 shows the ability of ATGs and $F(ab')_2$ fragments thereof to bind to human complement.

Figure 9. ATG $F(ab')_2$ fragments do not induce complement-dependent lysis of human PBLs and PHA-activated PBLs. Figure 9 shows the ability of ATGs and $F(ab')_2$ fragments thereof to induce complement-dependent lysis of human PBLs and PHA-activated PBLs.

Figure 10. ATG $F(ab')_2$ fragments do not induce antibody-dependent cell cytotoxicity (ADCC) of PHA-activated PBLs. Figure 10 shows the ability of ATGs and $F(ab')_2$ fragments thereof to induce ADCC-mediated lysis of PHA-activated PBLs.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based at least in part on the novel and surprising finding that $F(ab')_2$ and Fab fragments of polyclonal antilymphocyte globulin (ALG) and polyclonal antithymocyte globulin (ATG) preparations, while incapable of promoting cell lysis due to the absence of F_c domains of the immunoglobulins, retain immunomodulatory activity. Thus, we herein provide a means by which one may obtain a desired immunomodulatory activity without

subjecting a host to the adverse massive lymphocytopenia that is observed with treatment with intact polyclonal ALGs/ATGs.

In this regard, one embodiment of the present invention is directed to a method for modulating a lymphocyte-mediated immune response which comprises contacting lymphocytes with an immunomodulating amount of $F(ab')_2$ and/or Fab fragments derived from a polyclonal antilymphocyte globulin preparation, wherein the immune response is modulated. For purposes herein, a "lymphocyte-mediated immune response" is an immune reaction that involves activated lymphocytes, particularly activated cytotoxic T lymphocytes, and their cytotoxic activities in a normal physiological environment.

For purposes of the present invention, the $F(ab')_2$ and Fab fragments which find use in the present invention may be obtained from virtually any polyclonal antilymphocyte antibody preparation, particularly an anti-cytotoxic T lymphocyte antibody preparation, wherein those antibodies may be antibodies that are originally present in polyclonal antilymphocyte globulin (ALG) or antithymocyte globulin (ATG) preparations that are prepared in animals other than humans, particularly horse or rabbit. In this regard, "antilymphocyte antibodies" are antibodies that bind specifically to one or more protein(s) on the surface of lymphocytes, preferably proteins which are specifically expressed on the surface of lymphocyte cells such as, for example, CD1, CD2, CD3, CD4, CD5, CD6, CD7, CD8, CD11, CD16, CD18, CD20, CD28, CD38, CD40, CD44, CD45, CD56, CD58 and the T cell receptor.

Polyclonal antilymphocyte globulin (ALG) and antithymocyte globulin (ATG) preparations are well known in the art as being useful for immunosuppressive applications in humans. ALGs/ATGs are for the most part prepared in horses or rabbits, wherein one immunizes the host animal with human lymphocytes or T cells and

then isolates a polyclonal preparation of immunoglobulins from the host animal that contains IgG antibodies directed against human lymphocyte or T cell surface proteins. ALG and ATG preparations are readily commercially available and/or may be readily produced and purified using well known methodology such as that described by Rebellato et al., *Transplantation* 57:685-694 (1994), the disclosure of which is incorporated herein by reference.

Once obtained, polyclonal ALG and/or ATG preparations are then subjected to known techniques for obtaining purified Fab and $F(ab')_2$ fragments therefrom. Specifically, ALG and ATG polyclonal antibody preparations may be subjected to treatment with effective amounts of papain or pepsin and then subjected to column chromatography for the isolation of Fab or $F(ab')_2$ fragments, respectively. Techniques for obtaining both Fab and $F(ab')_2$ fragments from polyclonal antibody preparations are well known in the art (see, e.g., Abbas et al., *Cellular and Molecular Immunology*, 2nd ed., W.B. Saunders Company (1994) and the Examples below) and can be readily employed for the preparation of both the Fab and $F(ab')_2$ antibodies that find use herein.

The Fab and/or $F(ab')_2$ antibody fragments prepared and isolated as described above may be employed *in vitro*, *in vivo* and *ex vivo* for immunomodulatory purposes. Specifically, lymphocytes may be contacted *in vitro* with the Fab and/or $F(ab')_2$ fragments described herein for various purposes including, for example, for analyzing mechanisms underlying antibody-induced immunosuppression or immunoactivation. The subject antibody fragments can be also used *in vitro* to inhibit lysis by effector cells of target antigen presenting cells. Thus, in research where one wishes to maintain mixtures of cells, where cytotoxic lymphocytes would be activated and kill antigen presenting cells, such as macrophages or B-lymphocytes, or other cells which might serve as

target cells, e.g., neoplastic cells, viral infected cells, or the like, the lysis can be inhibited so that the cellular population may be maintained while under investigation.

In addition to the above described *in vitro* applications, the subject Fab and $F(ab')_2$ fragments may also be employed *in vivo* and *ex vivo* for a variety of different applications. Preferably, Fab fragments and/or submitogenic amounts of $F(ab')_2$ fragments that exhibit immunosuppressive activity may be employed for suppressing an immune response in a mammal, wherein the immune response may be associated with a variety of different adverse conditions including, for example, autoimmune diseases such as insulin-dependent diabetes mellitus (IDDM), rheumatoid arthritis, colitis, multiple sclerosis, and the like, Alzheimer's disease, Crohn's disease, asthma, septic shock, etc. In fact, the Fab and $F(ab')_2$ fragments described herein may find use for immunosuppression in virtually any disorder that is associated with an adverse immune response. Since the mechanism by which the subject Fab and $F(ab')_2$ fragments applies to lymphocytes in general, these fragments would be expected to be useful for immunomodulation in virtually any application where immunomodulation is desired.

In a particularly preferred embodiment, Fab fragments and submitogenic concentrations of $F(ab')_2$ fragments of the present invention that exhibit immunosuppressive activity may be employed for the prevention of graft rejection in transplant patients, for the reversal of ongoing graft rejection, and/or for the induction of tissue graft acceptance. For purposes of the present invention, the terms "graft" or "tissue graft" refer to any tissue that may be transplanted into a mammalian host, including solid organs, cells, and the like, and which may potentially give rise to an adverse immunological reaction in the recipient. For the purposes of the present invention and for the desired application, Fab and/or $F(ab')_2$ fragments may be

administered to the host either before or after the graft is introduced into the host or both.

The subject Fab fragments and submitogenic amounts of $F(ab')_2$ fragments may also be used *ex vivo*. In cases of transplantation of organs, particularly solid organs or particular cells, whether xenogeneic or allogeneic, the donor organ may be bathed in a medium comprising the subject antibody fragments. In this way, cytotoxic lymphocytes present within the implant will be inhibited from participating in graft versus host disease. Also, during the period when the subject antibody fragments remain bound to the implant, the recipient's lymphocytes will be inhibited from being activated. Generally, the concentration of the antibody fragments will vary in the medium, depending upon the activity of the fragments, the level of inhibition desired, the presence of other compounds affecting lymphocyte activation, and the like. Other immunosuppressants which may be present include cyclosporin A, FK506, and the like. Subtherapeutic dosages will be employed, generally when present, not less than about 1% of the normal dosage, and not more than about 75%, usually in the range of about 5 to 50%. Other components of the bathing medium will generally be constituents normally used in an organ preservation solution, e.g. HBSS. The time for the organ to be maintained in the medium will generally be in the range of about 2 to 72 hours.

As described above, the subject compositions may be employed *in vivo*, administrating the subject antibody fragments by any convenient means. The subject fragments may be administered prior to implantation, administration usually beginning not earlier than about 14 days prior to implantation, there preferably being at least one dosage administered within three days of implantation. The subject fragments may be administered in the period beginning about 6 hours prior to implantation and may be continued on a

predetermined schedule thereafter, usually not past 30 days, more usually not past 20 days. However, after implantation, the subject antibody fragment compositions may be administered as needed, depending upon the response of the recipient to the organ or cells. In some situations, the subject compositions may be administered chronically, as long as the implant is present in the host.

Generally, in the case where a Fab and/or $F(ab')_2$ composition is administered directly to a host, a bolus of the subject composition that is administered will be determine empirically. The host may be any mammal including domestic animals, pets, laboratory animals, primates, particularly humans. The amount administered will generally be adjusted depending upon the half life of the antibody fragments, where the half life will generally be at least ten minutes, more usually at least about 60 minutes, desirably at least about 12 hours, more desirably at least about 24 hours, preferably at least about 120 hours and more preferably at least about 240 hours. Short half-lives are acceptable, so long as efficacy can be achieved with individual dosages or continuous infusion or repetitive dosages. Dosages in the lower portion of the acceptable range and even lower dosages may be employed, where the antibody fragments have an enhanced half life or is provided as a depot, such as a slow release composition comprising particles, introduced in a matrix which maintains the fragments over an extended period of time, e.g., a collagen matrix, use of a pump which continuously infuses the fragments over an extended period of time with a substantially continuous rate, or the like.

As described above, transplantation may involve any organ or cells, including organs such as a heart, kidneys, lung, eyes, liver, gut, vascular vessel, or other organ, and cells, such as β-islet cells, bone marrow cells, or other cells, where the organ or cells are allogeneic or xenogeneic, particularly where one or more of the

Class I or II MHC antigens are different in the donor as compared to the recipient.

The subject Fab and/or $F(ab')_2$ antibody fragments, by themselves or as conjugates, may be prepared as formulations in pharmaceutically acceptable media, for example, saline, PBS, aqueous ethanol, glucose, propylene glycol, or the like or as solid formulations in appropriate excipients, generally at a pharmacologically effective dose. The concentrations or effective immunomodulatory amounts of the fragments will be determined empirically in accordance with conventional procedures for the particular purpose. The formulations may include bactericidal agents, stabilizers, buffers, or the like. The amount administered to the host will vary depending upon what is being administered, the purpose of the administration, such as prophylaxis or therapy, the state of the host, the manner of administration, the number of administrations and the interval between administrations, and the like, and such may be determined empirically by those skilled in the art. In order to enhance the half life of the subject antibody fragments, the fragments may be encapsulated, introduced into the lumen of liposomes, prepared as a colloid, or other conventional technique may be employed, which provides an extended life time of the fragments *ex vivo* or *in vivo*.

Depending upon their intended use, particularly for administration to mammalian hosts, the subject Fab and/or $F(ab')_2$ fragments may be modified widely to change their distribution in the blood stream, diminish or enhance binding to blood components, enhance the lifetime of the fragment in the blood stream, and the like. The subject antibody fragments may be bound to these other components by linkers which are cleavable or non-cleavable in the physiological environment of the blood. The fragments may be joined at any point where a functional group is present, such as

hydroxyl, thiol, carboxyl, amino, or the like. Desirably, binding will be at either the N-terminus or the C-terminus. The antibody fragments may also be joined to a wide variety of other oligopeptides or proteins for a variety of purposes.

An additional embodiment of the present invention is directed to a method for activating a lymphocyte-mediated immune response which comprises contacting lymphocytes with a mitogenic amount of $F(ab')_2$ fragments of antilymphocyte immunoglobulins, wherein the lymphocyte-mediated immune response is thereby activated. As shown in the Examples below, Fab fragments of antilymphocyte immunoglobulins function to block the activation of human lymphocytes and do not bind to complement or F_c receptors. As a result, Fab fragments exhibit primarily an immunosuppressive effect at virtually any concentration employed. Immunomodulatory concentrations or amounts and immunosuppressive amounts or concentrations of the Fab fragments, therefore, which find use herein may be readily determined in an empirical manner.

As also demonstrated in the Examples presented below, while $F(ab')_2$ fragments do not bind to complement or F_c receptors, they are capable of fully activating T lymphocytes at mitogenic concentrations whereas at submitogenic concentrations, incomplete T cell activation occurs. One skilled in the art may readily determine mitogenic (i.e., immunoactivating) and submitogenic (i.e., immunosuppressive) concentrations of the $F(ab')_2$ fragments, wherein those concentrations would expect to vary depending upon the antibody fragment being employed and the desired application. For the most part, however, mitogenic concentrations of $F(ab')_2$ fragments will be expected to be at least about 75 μg/ml, preferably at least about 100 μg/ml and more preferably at least about 150 μg/ml, whereas submitogenic concentrations will generally be less than about 75 μg/ml, usually less than about 50 μg/ml.

Genetically engineered animals that express human immunomodulatory Fab and $F(ab')_2$ antibody fragments or intact whole antibodies that do not bind to complement or F_c receptors and are therefore non-lytic, are also provided herein. In this regard, techniques for introducing nucleic acid encoding functional human proteins into the genome of non-human animals such as mice or rabbits is well known in the art and may be employed herein to produce genetically engineered animals that express non-lytic, antilymphocyte, immunomodulatory Fab and $F(ab')_2$ antibody fragments or intact antilymphocyte antibodies. Specifically, one skilled in the art may readily replace the IgG genes of an animal with nucleic acid encoding the desired human antibody or antibody fragments, thereby provide the desired genetically engineered animal.

In regard to the above, another embodiment of the present invention is directed to a composition of matter that comprises an antilymphocyte antibody that does not substantially bind to complement or F_c receptors, yet surprisingly retains immunomodulatory activity. As shown for the first time in the present application, at least a portion of the immunomodulatory activity of antilymphocyte antibodies is not associated with either the complement or F_c binding functions of such antibodies. Thus, one may now obtain antibodies that lack complement and F_c receptor binding activity, yet retain immunomodulatory activity. In this regard, it is noted that techniques for introducing mutations into intact antibodies so as to reduce or eliminate their ability to bind to F_c receptors and/or complement are known (see, e.g., Cole et al., *J. Immunol.* 159(7):3613-3621 (1997) and *Encyclopedia of Immunology*, eds. Roitt et al., Academic Press Inc, San Diego (1992)) and could be employed to obtain the claimed antibodies. In

a particularly preferred embodiment, the antilymphocyte antibody is directed against a T lymphocyte cell surface protein.

Further details of the invention are illustrated in the following non-limiting examples.

EXPERIMENTAL

EXAMPLE I - Preparation of ATG fragments.

ATGs used for the following experiments were produced in both rabbits and horses and were purified using known techniques. F(ab')2 and Fab fragments of ATGs were then prepared by pepsin or papain digestion, respectively, and purified by exclusion on protein A-Sepharose chromatography as previously described (Weir, *Handbook of Experimental Immunology-Immunochemistry*, vol. 1 (ed. 4), Oxford, UK, Blackwell Scientific, 1986). Briefly, for the isolation of $F(ab')_2$ fragments, ATGs were dialyzed against 0.1M acetate buffer (pH 4.5) and then the concentration of ATGs was measured at A_{280}, wherein the ATG concentration should be about 20-30 mg/ml. Pepsin was then dissolved in 0.1M acetate buffer (pH 4.5) and was added to the antibody with a ratio E/A of 1:200. The reaction mixture was incubated for 18 hours at 37°C and the reaction was then stopped by addition of 1M NaOH. The pH should be around 8.0. The mixture was then dialyzed against PBS and loaded on protein A-Sepharose CL-4B column. The unbound fraction was collected.

For the isolation of Fab fragments from the ATG preparation, the ATGs are dialyzed against phosphate-buffered saline (PBS) pH 7.4 and incubated with activated papain at a ratio of 1:20 to 1:200. Following incubation at 37°C, the reaction was stopped by the addition of iodoacetamide. The mixture was then dialyzed against

PBS and loaded on protein A-Sepharose CL-4B column. The unbound fraction was collected.

EXAMPLE 2 - ATG F(ab')$_2$ and Fab fragments bind to a variety of lymphoid cell surface antigens.

Competition experiments employing monoclonal antibodies and immunoprecipitation experiments demonstrated that ATG preparations contain antibodies specific for a wide range of different cell surface molecules, many of which are expressed exclusively on T cells. Similarly, ATG F(ab')$_2$ and Fab fragments prepared as described in Example 1 above compete with the binding of monoclonal antibodies to a variety of lymphocyte cell surface antigens (data not shown) and, therefore, are also directed to a wide variety of different cell surface antigens, including T cell antigens. This is due to the fact that the antibody specificity of ATGs and F(ab')2 and Fab fragments prepared from those ATGs are identical.

EXAMPLE 3 - ATG F(ab')$_2$ fragments activate human peripheral blood lymphocytes (PBLs).

Oligomerization of T cell receptor/CD3 (TCR/CD3) molecules of T cells induces activation signals resulting in cytokine gene expression, synthesis of CD25 (the α chain of the IL-2 receptor) and T cell proliferation. TCR/CD3 oligomerization can be induced by the anti-CD3 antibody OKT3, providing that OKT3 antibodies are cross-linked by engagement with Fc receptors on accessory cells such as monocytes.

To determine if ATGs and/or their associated F(ab')$_2$ and Fab fragments are capable of activating human PBLs, the following experiments were carried out. Specifically, PBLs were prepared from peripheral blood mononuclear cells (PBMCs) by the elimination

of monocytes and natural killer cells by L-leucine methyl ester treatment (Thiele et al., *Eur. J. Immunol.* 131:2282 (1983)). Monocyte depletion was assessed by flow cytometric analysis after staining with LEU M3 (CD14) monoclonal antibody (Becton Dickinson, Pont de Claix, France). PBMCs or PBLs were then incubated in the presence of medium alone (med), 100 μg/ml rabbit ATG prepared as described above (ATG), 100 μg/ml of F(ab')$_2$ fragments derived from the rabbit ATG preparation (F(ab')$_2$), 100 μg/ml of antibody OKT3 (Orthoclone), 5 μg/ml PHA (Sigma Chemical Co., St. Louis, MO) or 10 ng/ml phorbol myristate acetate (PMA) plus 1 μg/ml ionomycin (Sigma Chemical Co., St. Louis, MO) (P/I). Cultures were maintained in a humidified atmosphere containing 5% CO_2. The cells were pulsed with 0.5 μCi/well of ^{3}H TdR (CEA, Saclay, France) during the last 8 hours of a 72 hour culture period. The results of these experiments are shown in Figure 1.

As shown in Figure 1, ATGs and their F(ab')2 fragments can induce T cell activation *in vitro*, as demonstrated by an observed increase in DNA synthesis. However, no *in vitro* T cell activation was observed using ATG Fab fragments (data not shown). Decreasing the percentage of monocytes in the cell suspension markedly decreases the proliferative response to OKT3 and PHA but does not affect the response to ATGs. These results demonstrate that both ATGs and F(ab')$_2$ fragments thereof are able to crosslink TCR/CD3 molecules to an extent resulting in T cell stimulation. This crosslinking capability is independent of interaction with monocyte Fc receptors and accounts for the mitogenic activity of F(ab')$_2$ fragments. The lack of T cell stimulation by Fab fragments is due to their inability to crosslink TCR/CD3 molecules. In this regard, it is noted that we previously reported that the mitogenic activity exhibited by ATGs was associated with the presence of anti-CD3

and anti-CD2 antibody specificities in the ATGs (Bonnefoy-Bérard et al. (1991), *supra*).

EXAMPLE 4 - At submitogenic concentrations, ATG F(ab')$_2$ fragments induce incomplete T cell activation.

To determine whether ATG F(ab')$_2$ fragments are capable of fully inducing T cell activation, the following experiment was performed. Specifically, PBLs were cultured in the presence of a rabbit ATG preparation or F(ab')$_2$ fragments derived therefrom at 10 or 100 μg/ml for 3 days. At days 0, 1, 2 and 3, T cell surface expression of CD69, CD95 and CD25 was determined by flow cytometry. The results of these experiments are expressed as mean fluorescence intensity (MFI, arbitrary units) and are shown in Figure 2.

As shown in Figure 2, while a concentration of 100 μg/ml causes T cell activation, ATG F(ab')$_2$ fragments at a submitogenic concentration of 10 μg/ml induce only incomplete T cell activation, demonstrated by the expression of T cell surface markers of the G1 phase of the cell cycle, but little or no cell proliferation. PBLs incubated with ATG F(ab')$_2$ fragments express the early activation markers CD69 and CD95 (Fas), undergo chromatin decondensation and cell size enlargement (assessed by flow cytometry) but do not express CD25 and, therefore lack the trimeric interleukin-2 (IL-2) receptor which is required for IL-2 dependent progression into the S phase of the cell cycle. As expected, addition of exogenous human recombinant IL-2 does not restore the T cell proliferation (data not shown).

In addition, ATG F(ab')$_2$ fragments induce the expression of surface CD95 (Fas, Apo-1) and trigger CD95-L mRNA and protein synthesis by human PBL (data not shown).

<u>EXAMPLE 5</u> - ATG F(ab′)$_2$ fragments down-regulate surface expression of lymphocyte surface molecules that control T cell activation.

The immunosuppressive anti-CD3 antibody OKT3 activates T cells *in vivo*, inducing a massive release of IL-2, TNFα and IFNγ, which results in the so-called first injection syndrome (fever, malaise, hypotension, headache, etc.). However, subsequent injections do not induce any T cell activation effects because the TCR/CD3 complexes have been internalized and are no longer expressed at the cell surface. This effect is referred to as "modulation" of TCR/CD3. Modulation results in T cell unresponsiveness to any activation signal that depends on TCR/CD3 oligomerization and it accounts for the potent immunosuppressive activity of OKT3.

Knowing that ATG F(ab′)$_2$ fragment preparations contain various antibodies specific for a wide array of T cell surface molecules, we investigated whether ATG F(ab′)$_2$ fragments could induce modulation of surface molecules that play a major role in the triggering or the control of T cell activation. Specifically, PBMCs were incubated with a rabbit ATG preparation or F(ab′)$_2$ fragments derived therefrom at 10 and 100 μg/ml for 10 minutes at 4°C, washed twice and then cultured in the presence of medium alone at 37°C in humidified atmosphere containing 5% CO_2. Expression of CD3, TCR, CD4, CD8, CD2 and CD11a molecules at 0, 2, 4, 12 and 24 hours of culture was measured using specific FITC-labeled monoclonal antibodies. The results as shown in Figure 3 are expressed as specific MFI calculated according to the following formula: specific MFI = 100 - (test MFI x 100) / control MFI, where control MFI represents MFI observed after incubation at 4°C

in the presence of medium alone and test MFI is the MFI observed after incubation at 37°C.

As shown in Figure 3, ATG $F(ab')_2$ fragments induce a marked decrease of surface expression of the antigen receptors CD3/TCR, the co-receptors CD2, CD4, CD8 and the integrin CD18/CD11a.

In addition to OKT3, other antibodies (e.g. anti-CD2, anti-CD4, anti-CD8, anti-CD11a) can induce modulation of the corresponding surface antigens but this effect is always restricted to the molecule that is specifically recognized by the antibody. Furthermore, it usually follows slower kinetics than that observed with ATG $F(ab')_2$ fragments and, depending on the antibody IgG subclass, may or may not require cross-linking through binding to monocyte Fc receptors. Remarkably, we show herein that modulation can be achieved by ATG $F(ab')_2$ fragments, indicating that cross-linking by accessory cells is not required.

Determination of the antibody specificities in ATG $F(ab')_2$ or Fab fragments preparations can be performed by competition with various labeled monoclonal antibodies and measurement of the decrease of fluorescence signal in flow cytometry analysis (this method was used in several reports including a study from our group: Bonnefoy-Bérard, (1991), *supra*)). Using this method, a large number of antibody specificities have been characterized in ATGs. In some studies, the results were confirmed by immunoprecipitation of T cell lysates and sodium dodecylsulfate polyacrylamide gel electrophoresis. The data obtained by competition experiments in flow cytometry may vary, depending on the choice of the monoclonal antibody used for competition (the results apply to the epitope recognized by the antibody but do not necessarily extend to other epitopes of the same molecules). Furthermore identification of an antibody specificity by competition experiments does not

necessarily imply that the antibody present in ATG can induce functional effects (depending on affinity, relative concentration, etc.). The modulation technique allows a precise and straightforward assay of all relevant functional antibody specificities.

The presence of functional antibody specificities in ATGs is usually matched by the presence of the matching antigen in the immunizing cell preparation (e.g. human thymocytes). Following the clinical experience acquired with the development of new immunosuppressive antibodies that do not induce lymphocyte depletion upon injection *in vivo*, it is possible to modify the cell preparation used for immunization in order to ensure the presence of the desired specificity in polyclonal ATGs and their $F(ab')_2$ or Fab fragments.

EXAMPLE 6 - Modulation of T cell surface receptors is associated with T cell unresponsiveness to mitogenic or antigenic stimuli.

The down-regulation of surface expression of the antigen/receptor complexes TCR/CD3 and that of the co-receptors CD2, CD4 and CD8 is expected to be associated with decreased responses to mitogenic stimuli. To confirm this, we carried out the following experiments. Specifically, PBMCs were cultured in the presence of medium alone, rabbit ATGs or $F(ab')_2$ fragments derived therefrom at 100 μg/ml for 20 hours, then cells were washed twice and cultured for 72 hours in the presence of medium alone, OKT3 antibody (100 μg/ml) or PMA (10 ng/ml) plus ionomycin (1 μg/ml) (P/I). Cultures were maintained in a humidified atmosphere containing 5% CO_2. Cells were pulsed with 0.5 μCi/well of ^{3}H TdR (CEA, Saclay, France) during the last 8 hours of a 72 hour culture period. The results from these experiments are shown in Figure 4.

As shown in Figure 4, under the above described conditions, a marked hyporesponsiveness is observed, whereas proliferative responses to a phorbol ester (PMA) associated with the calcium ionophore ionomycin, which bypasses initial activation signals from the TCR/CD3 complex by directly increasing intracellular calcium and activating protein kinase C, is not affected by modulation. This indicates that modulated T cells have become anergic and refractory to signals generated by the TCR but have not been killed by the antibodies.

These observations are further extended to mixed lymphocyte reactions, an *in vitro* model of T cell response to allogeneic grafts, in which normal PBLs are activated by allogeneic cells (from a human B cell line). Specifically, PBMCs were cultured in the presence of RAJI cells which are used as stimulators. Stimulator cells were treated for 1 hour at 37°C with 25 μg/ml of mitomycin C, extensively washed, then mixed with PBMCs at a ratio of one B cell for 20 PBMCs in the presence of medium alone, rabbit ATGs or $F(ab')_2$ fragments derived therefrom at 10 μg/ml. DNA synthesis was measured after addition of 0.5 μCi/well of ^{3}H TdR during the last 8 hours of a 6 day culture period. The results are shown in Figure 5.

The results shown in Figure 5 show that ATG $F(ab')_2$ and intact ATGs at submitogenic concentrations inhibit transformation and proliferation of T cells significantly.

<u>EXAMPLE 7</u> - ATG $F(ab')_2$ fragments induce apoptosis of activated but not resting T cells.

To determine whether ATG and fragments thereof are capable of inducing apoptosis of T cells, the following experiment was carried out. specifically, PBLs were cultured in the presence of medium alone or PHA (5 μg/ml) for 3 days. Dead cells were

removed by centrifugation on a layer of Histopaque (Sigma) and viable cells were treated for 20 hours with rabbit ATGs, $F(ab')_2$ fragments derived therefrom, normal rabbit Ig at 10 μg/ml or the agonistic anti-Fas (CD95) antibody CH11 (Coulter Immunotech, Marseilles, France) at 1 μg/ml. The percentage of apoptotic cells was determined by fluorescence microscopy after staining with Hoechst 33342. Nuclear condensation or marked condensation of the chromatin with reduction of nuclear size, or both, were considered typical features of apoptotic cells. On the basis of these measurements, results were expressed as percentage of specific apoptosis according to the formula: % specific apoptosis = (test-control) x 100 / (100-control). The results of these experiments are shown in Figure 6.

Activated T cells express the apoptosis inducing receptor CD95 and can be killed by interaction of CD95 with CD95L. Expression of CD95L can be triggered by ATG $F(ab')_2$ fragments at mitogenic (100 mg/ml) and submitogenic (10 mg/ml) concentrations. As shown in Figure 6, ATG $F(ab')_2$ fragments induce apoptosis of PHA-activated PBLs but not of non-activated PBLs.

In vivo treatments with ATG $F(ab')_2$ fragments at doses which maintain plasma concentrations below the threshold of mitogenic activity but yet sufficient to trigger CD95L expression results in the induction of apoptosis in activated but not resting T cells. Thus, activated donor specific T cells after organ transplantation are eliminated following administration of ATG $F(ab')_2$ fragments. Similarly, activated tissue specific T cells in autoimmune disease patients are eliminated following administration of ATG $F(ab')_2$ fragments. Lastly, activated T cells causing graft-versus host disease following bone marrow transplantation are deleted by administration of ATG $F(ab')_2$ fragments. The deletion of

activated T cells in these clinical situations results in the acceptance of a transplanted organ or cells (induction of tolerance) and reverses autoimmune disease.

<u>EXAMPLE 8</u> - ATG Fab fragments block the activation of human PBLs.

ATG F(ab')2 and Fab fragments bind to a wide range of lymphocyte cell surface molecules. Several of these cell surface proteins are involved in signal transduction upon stimulation of T cells (for example: TCR, CD3, CD3, CD4, CD8, CD11, CD18, CD28, CD40, CD45). To determine if ATGs and/or fragments thereof are capable of inhibiting T cell proliferation, we carried out the following experiment. PBLs were prepared from human blood by Ficoll-Hypaque centrifugation and incubated with allogeneic mitomycin-C treated EBV transformed B cells as stimulators. ATG Fab or control Fab fragments were added to the culture at the concentrations indicated in Figure 7. Cell proliferation was measured by determination of ^{3}H TdR incorporation during the final 18 hours of a 102 hour incubation at 37°C in a humidified atmosphere containing 5% CO_2. Statistical analysis indicated a significant ($p<0.01$) reduction of proliferation at the ATG Fab concentrations ("a") indicated in Figure 7.

As shown in Figure 7, Fab fragment-mediated blockade of the above described cell surface molecules results in the prevention of T cell activation or incomplete activation of T cells. Specifically, in the absence of ATG Fab, T cells are activated and proliferate. In contrast, addition of ATG Fab results in a dose dependent inhibition of T cell proliferation.

<u>EXAMPLE 9</u> - Incomplete activation of human PBLs in the presence of ATG Fab results in the induction of apoptosis

Incomplete activation of human PBLs results in the induction of apoptosis. This is demonstrated by activation of a human T cell clone in the presence of ATG Fab fragments. In the presence of ATG Fab fragments, a large number of T cells undergo apoptosis. In the absence of an activation signal, ATG Fab fragments have no effect on T cell survival (data not shown).

In vivo treatments with ATG Fab fragments results in the blockade of costimulatory molecules and the subsequent induction of apoptosis in the incomplete activated T cells. In contrast, resting non-activated T cells are unaffected. Thus, activated donor specific T cells after organ transplantation may be eliminated following administration of ATG Fab fragments. Similarly, activated tissue specific T cells in autoimmune disease patients may be eliminated following administration of ATG Fab fragments. Lastly, activated T cells causing graft-versus host disease following bone marrow transplantation may be deleted by administration of ATG Fab fragments. The deletion of activated T cells in these clinical situations is expected to result in the acceptance of a transplanted organ or cells (induction of tolerance) and reverses autoimmune disease.

<u>EXAMPLE 10</u> - ATG F(ab')$_2$ and Fab fragments unlike intact ATGs do not interact with human complement.

Complement dependent lysis is a major pathway of lysis of antibody-coated cells in the circulation. The ability of ATGs and fragments thereof to bind to human C1q, the first component of the classical complement cascade was documented with both horse and rabbit ATGs by two following sets of experiments.

A. <u>ATG $F(ab')_2$ and Fab fragments do not bind human C1q</u>

PBLs and 3-day PHA-activated PBLs were labeled with rabbit ATGs, horse ATGs or their respective $F(ab')_2$ fragments at 1 mg/ml diluted in isotonic NaCl/Pi buffer containing 1% bovine serum albumin (BSA) and 0.2 % NaN_3 (PBS/BSA/Azide) for 30 min. at 37°C. After two washes in PBS, samples were separated in two and incubated at room temperature for 30 min. in the presence of 50 μl of autologous serum as a source of complement (solid line in Figure 8) or heat inactivated (56°C, 30 min.) serum (dashed line in Figure 8) as a control. After two washes, cells were incubated with 10 μl of polyclonal goat anti-C1q FITC antibody (1/50 Cappel, Durham, North Carolina) at 4°C for 30 minutes. After washes, cells were fixed with 1% formaldehyde in PBS/BSA/Azide buffer and analysis was performed on a FACScan flow cytometer. The results are shown in Figure 8.

As shown in Figure 8, rabbit and horse ATGs bind human C1q but $F(ab')_2$ fragments thereof do not. Similar results are observed with ATG Fab fragments (data not shown). As control, no significant C1q binding is observed when heat-inactivated serum (56°C, 30 min) is used instead of normal serum as a source of complement (data not shown).

B. <u>Whole ATG but not ATG $F(ab')_2$ or Fab fragments induce lymphocyte lysis in the presence of human serum</u>

In a second experiment, PBMCs were cultured for three days in the presence of medium alone or PHA (5 μg/ml), dead cells were removed by centrifugation on a layer of Histopaque (Sigma), and viable cells were labeled with $Na_2{}^{51}CrO_4$ for two hours at room temperature and washed twice. then cells were resuspended in medium at 2 x 10^6 cells per ml and 100 μl of the suspension was

added to round-bottomed microtiter plates containing 50 μl of an appropriate dilution of rabbit ATGs, horse ATGs or their respective $F(ab')_2$ fragments. After 10 min. at room temperature, 50 μl of 40 % fresh autologous serum was added. The cell suspensions were incubated at 37°C for 30 min., then centrifuged at 100g for 2 min., and 100 μl of the supernatant was collected for measurement of released radioactivity. Controls without antibody were used to measure the spontaneous radioactivity release. The percentage of specific ^{51}Cr release was calculated using the formula: specific release = (test-spontaneous) x 100 / (total-spontaneous). The results are shown in Figure 9.

As shown in Figure 9, chromium release in supernatant is measured as a highly sensitive indicator of cell lysis. The results of a typical experiment indicate that ATGs but not $F(ab')_2$ fragments induce lysis of PBL and PHA-activated PBL. Similar results are observed with Fab fragments (data not shown).

<u>EXAMPLE 11</u> - Rabbit ATGs but not ATG F(ab')2 or Fab fragments induce antibody dependent cell cytotoxicity of PHA-activated PBL.

Antibody dependent cell cytotoxicity (ADCC) is a cytotoxic assay which relies on the binding of the Fc region of antibodies (bound to ^{51}chromium labeled target cells) to Fc receptors of cytotoxic cells (e.g. NK cells in human peripheral blood) and triggers their cytotoxic activity. To determine if ATGs and/or fragments thereof are capable of inducing ADCC of PHA-activated PBLs, the following experiment was conducted. Specifically, PBMCs were activated for three days in the presence of PHA (5 μg/ml), dead cells were removed by centrifugation on a layer of Histopaque (Sigma), and viable cells were labeled with $Na_2{}^{51}CrO_4$ for 2 hours at room temperature and washed twice. Then cells were resuspended in medium at 1 x 10^6 cells/ml and 50 μl of the suspension was

added to round-bottom microtiter plates containing 50 μl of an appropriate dilution of rabbit ATGs, horse ATGs or their respective $F(ab')_2$ fragments. After incubation for 10 min. at room temperature, 100 μl of effector cells (fresh PBMC, 25 x 10^6 cells/ml) were added. The cell suspensions were incubated at 37°C for 6 hours then centrifuged at 100 x g for 2 min. and 100 μl of the supernatant was collected for measurement of released radioactivity as for complement-mediated lysis. The results as shown in Figure 10.

Cytotoxic indices are calculated as the specific chromium release (ratio E-S/T-S were E represents experimental cpm in the presence of ATGs, T cpm release by complete lysis of target cells and S spontaneous release). As shown in Figure 10, rabbit but not horse ATGs induce ADCC of PHA-activated PBL and $F(ab')_2$ fragments are inefficient. These data indicate that rabbit $F(ab')_2$ fragments unlike whole rabbit IgG do not bind to Fc receptors on human NK cells. Similar results are observed with ATG Fab (data not shown). These results demonstrate that ATG $F(ab')_2$ or Fab fragments do not induce massive lymphocytopenia because they lack the capacity to activate human complement and to bind to Fc receptors.

Concluding Remarks:

The foregoing description details specific methods which can be employed to practice the present invention. Having detailed such specific methods, those skilled in the art will well enough known how to devise alternative reliable methods at arriving at the same information in using the fruits of the present invention. Thus, however, detailed the foregoing may appear in text, it should not be construed as limiting the overall scope thereof; rather, the ambit of the present invention is to be determined only by the lawful

construction of the appended claims. All documents cited herein are expressly incorporated by reference.

WHAT IS CLAIMED IS:

1. A method for modulating a lymphocyte-mediated immune response, said method comprising:

contacting lymphocytes with an immunomodulating amount of a composition comprising fragments of a polyclonal antilymphocyte globulin (ALG) preparation, wherein said fragments are selected from the group consisting of $F(ab')_2$ and Fab fragments,

wherein said immune response is modulated.

2. The method according to Claim 1, wherein said polyclonal ALG preparation is a polyclonal antithymocyte globulin (ATG) preparation.

3. The method according to Claim 1, wherein said ALG preparation is obtained from an animal selected from the group consisting of rabbit and horse that was previously immunized with human lymphocytes.

4. The method according to Claim 1, wherein said ALG preparation is obtained from an animal selected from the group consisting of rabbit and horse that was previously immunized with cells genetically engineered to express on their surface at least one human lymphocyte cell surface protein.

5. The method according to Claim 1, wherein said step of contacting is in the presence of a viable solid organ or cells.

6. The method according to Claim 1, wherein said step of contacting occurs *in vitro*.

7. The method according to Claim 1, wherein said lymphocytes comprise cytotoxic T lymphocytes.

8. The method according to Claim 1, wherein said fragments are $F(ab')_2$ fragments.

9. The method according to Claim 1, wherein said fragments are Fab fragments.

10. A method for suppressing an immune response in a mammal, said method comprising:

administering to said mammal an immunosuppressing amount of a composition comprising fragments of a polyclonal antilymphocyte globulin (ALG) preparation, wherein said fragments are selected from the group consisting of $F(ab')_2$ and Fab fragments,

wherein said immune response is suppressed.

11. The method according to Claim 10, wherein said polyclonal ALG preparation is a polyclonal antithymocyte globulin (ATG) preparation.

12. The method according to Claim 10, wherein said ALG preparation is obtained from an animal selected from the group consisting of rabbit and horse that was previously immunized with human lymphocytes.

13. The method according to Claim 10, wherein said ALG preparation is obtained from an animal selected from the group consisting of rabbit and horse that was previously immunized with cells genetically engineered to express on their surface at least one human lymphocyte cell surface protein.

14. The method according to Claim 10, wherein said immune response is associated with lymphocyte-mediated rejection of a tissue graft transplanted into said mammal or an autoimmune disease.

15. The method according to Claim 14, wherein said step of administering is performed after said tissue graft is transplanted into said mammal.

16. The method according to Claim 14, wherein said step of administering is performed before said tissue graft is transplanted into said mammal.

17. The method according to Claim 14, wherein said step of administering is performed both before and after said tissue graft is transplanted into said mammal.

18. The method according to Claim 14, wherein said tissue graft comprises an intact organ or cells.

19. The method according to Claim 14, wherein said autoimmune disease is selected from the group consisting of insulin dependent diabetes mellitus, rheumatoid arthritis and multiple sclerosis.

20. The method according to Claim 10, wherein said immune response is associated with septic shock, colitis, Crohn's disease, Alzheimer's disease or asthma.

21. The method according to Claim 10, wherein said fragments are $F(ab')_2$ fragments.

22. The method according to Claim 10, wherein said fragments are Fab fragments.

23. A method for activating a lymphocyte-mediated immune response, said method comprising:

contacting lymphocytes with an immunoactivating amount of $F(ab')_2$ fragments of a polyclonal antilymphocyte globulin (ALG) preparation,

wherein said immune response is activated.

24. The method according to Claim 23, wherein said ALG preparation is a polyclonal antithymocyte globulin (ATG) preparation.

25. The method according to Claim 23, wherein said ALG preparation is obtained from an animal selected from the group consisting of rabbit and horse that was previously immunized with human lymphocytes.

26. The method according to Claim 23, wherein said ALG preparation is obtained from an animal selected from the group consisting of rabbit and horse that was previously immunized with cells genetically engineered to express on their surface at least one human lymphocyte cell surface protein.

27. A composition of matter comprising an antilymphocyte antibody that does not substantially bind to either complement or an F_c receptor, wherein said antibody exhibits immunomodulatory activity.

28. The composition of matter according to Claim 27, wherein said antibody binds to a T lymphocyte cell surface protein.

FIGURE 1

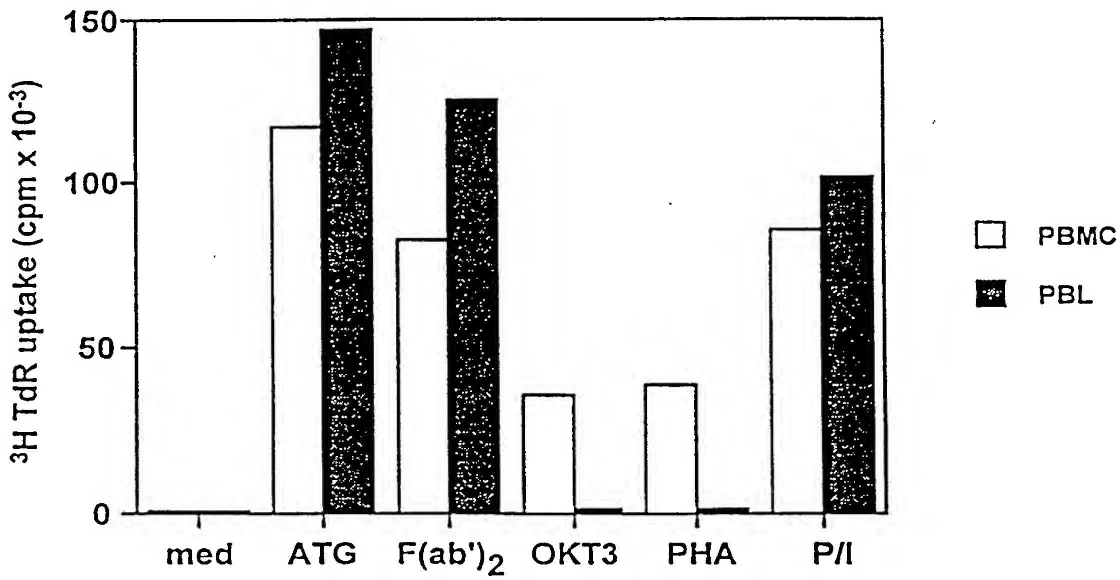

150
100
50
0
3H TdR uptake (cpm x 10-3)
med
ATG
F(ab')2
OKT3
PHA
P/I
PBMC
PBL

FIGURE 2

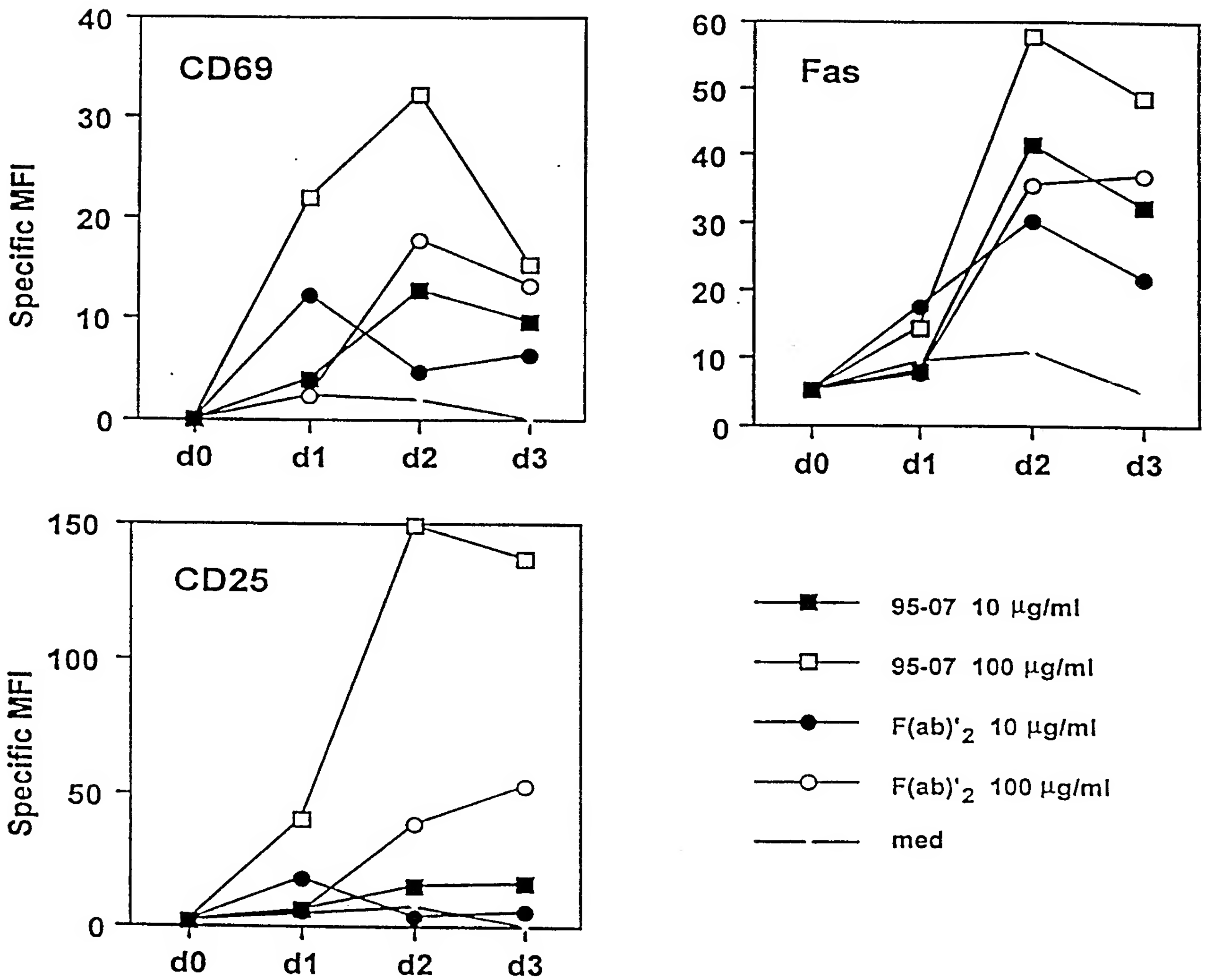

CD69
Specific MFI
40
30
20
10
0
d0
d1
d2
d3
Fas
60
50
40
30
20
10
0
d0
d1
d2
d3
CD25
Specific MFI
150
100
50
0
d0
d1
d2
d3
95-07 10 μg/ml
95-07 100 μg/ml
F(ab)'2 10 μg/ml
F(ab)'2 100 μg/ml
med

FIGURE 3

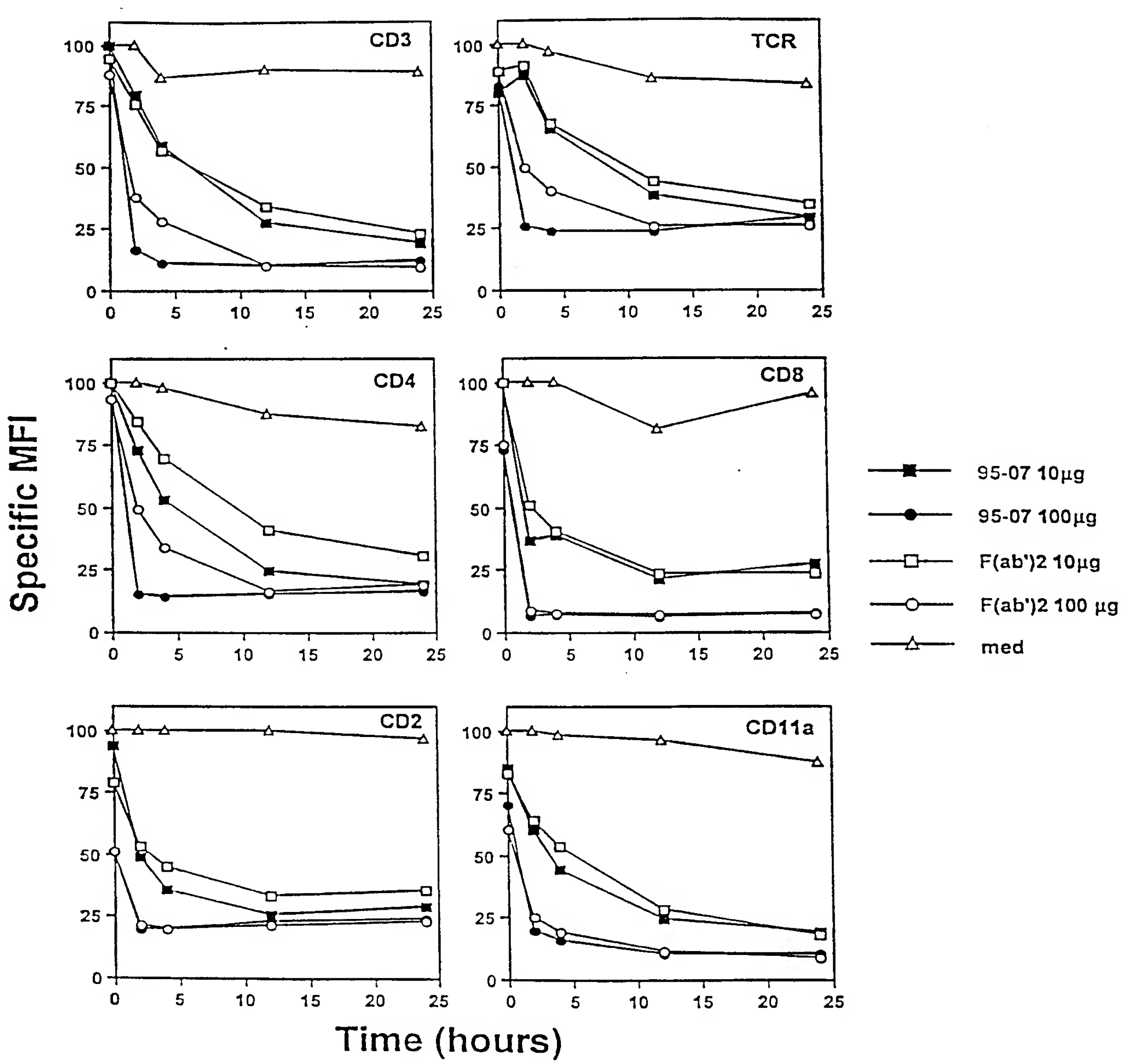

CD3
TCR
CD4
CD8
CD2
CD11a
Specific MFI
Time (hours)
95-07 10µg
95-07 100µg
F(ab')2 10µg
F(ab')2 100 µg
med

FIGURE 4

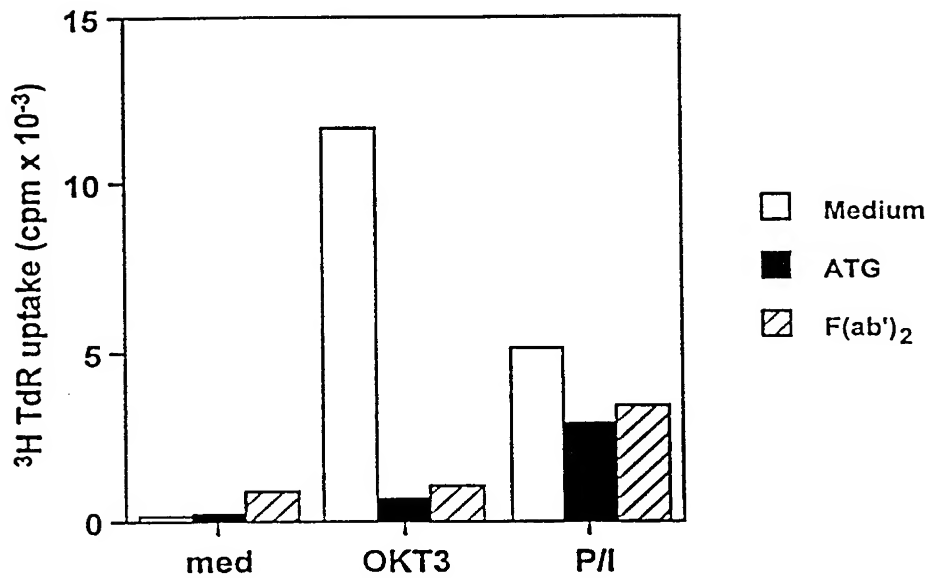


FIGURE 5

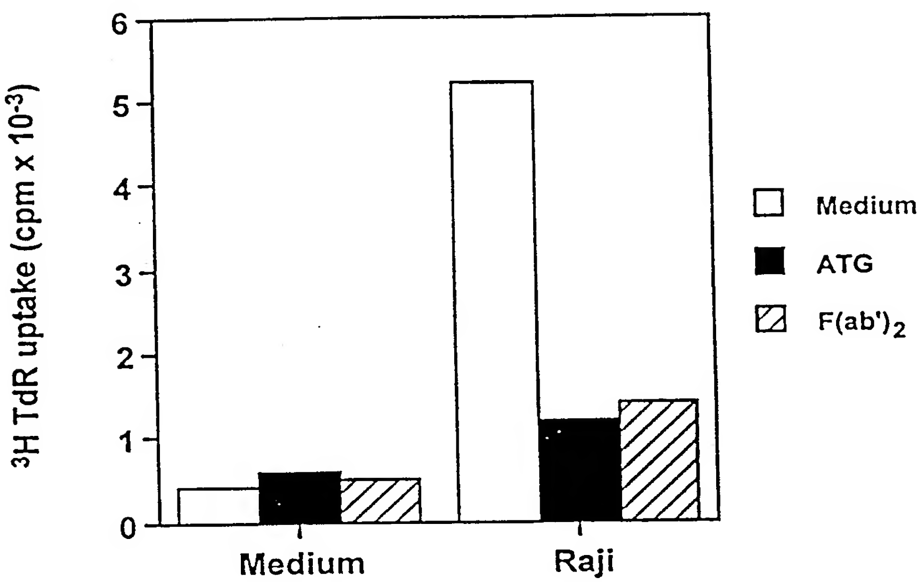


FIGURE 6

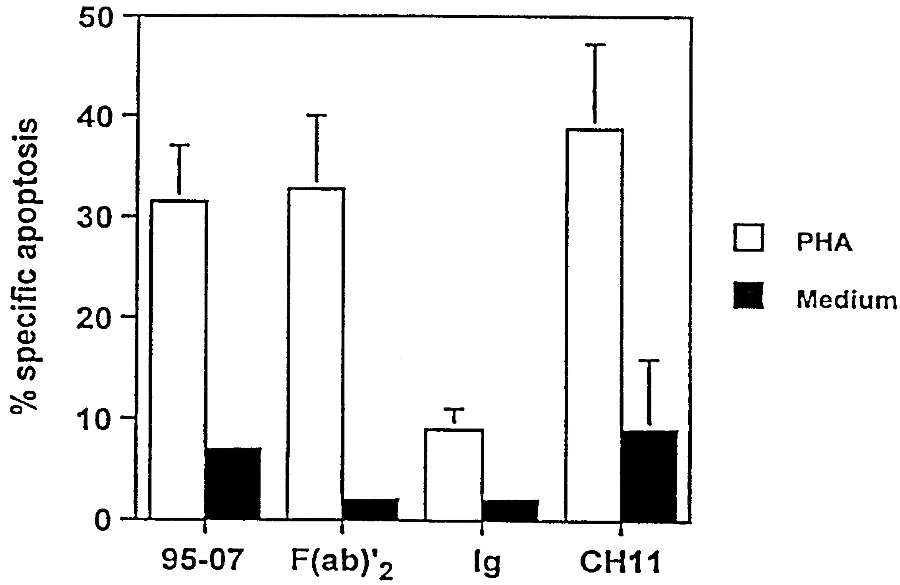

50
40
30
20
10
0
% specific apoptosis
95-07
F(ab)'2
Ig
CH11
PHA
Medium

FIGURE 7

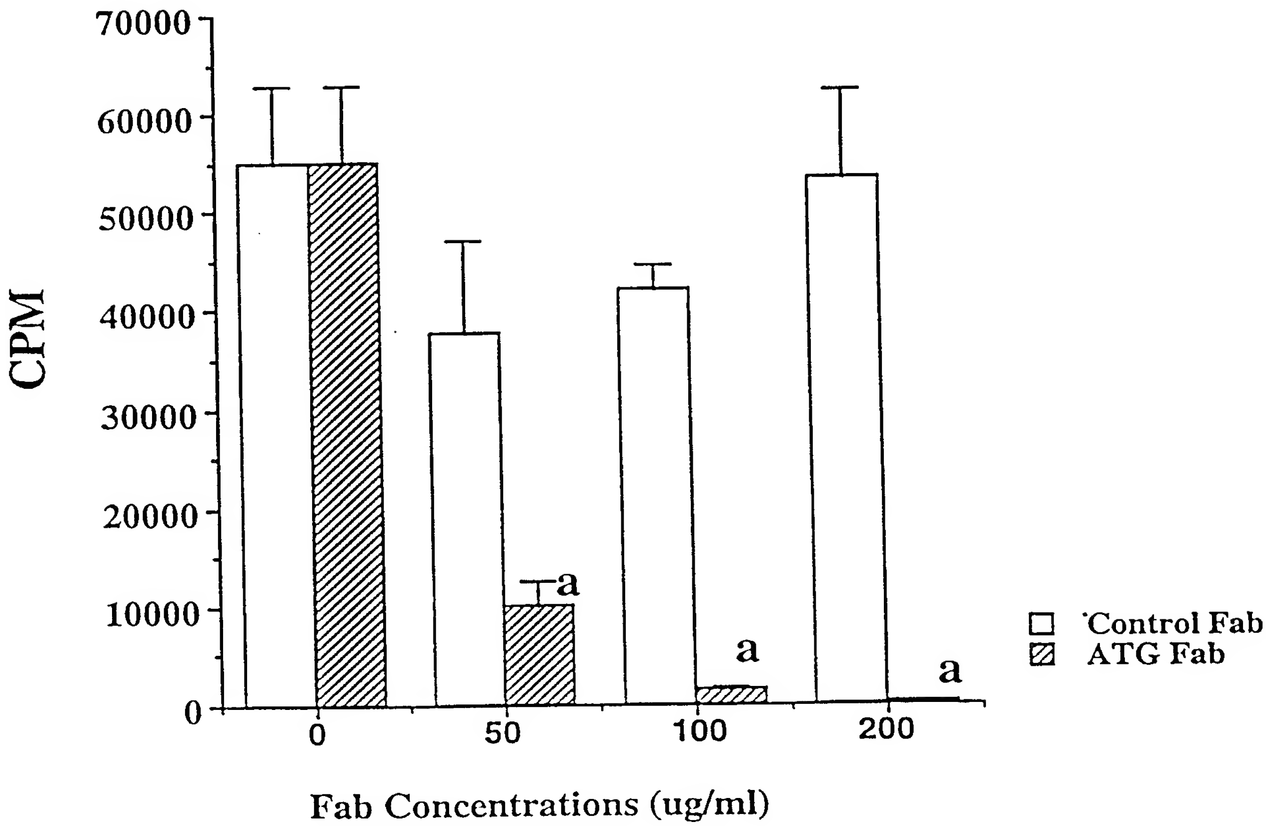
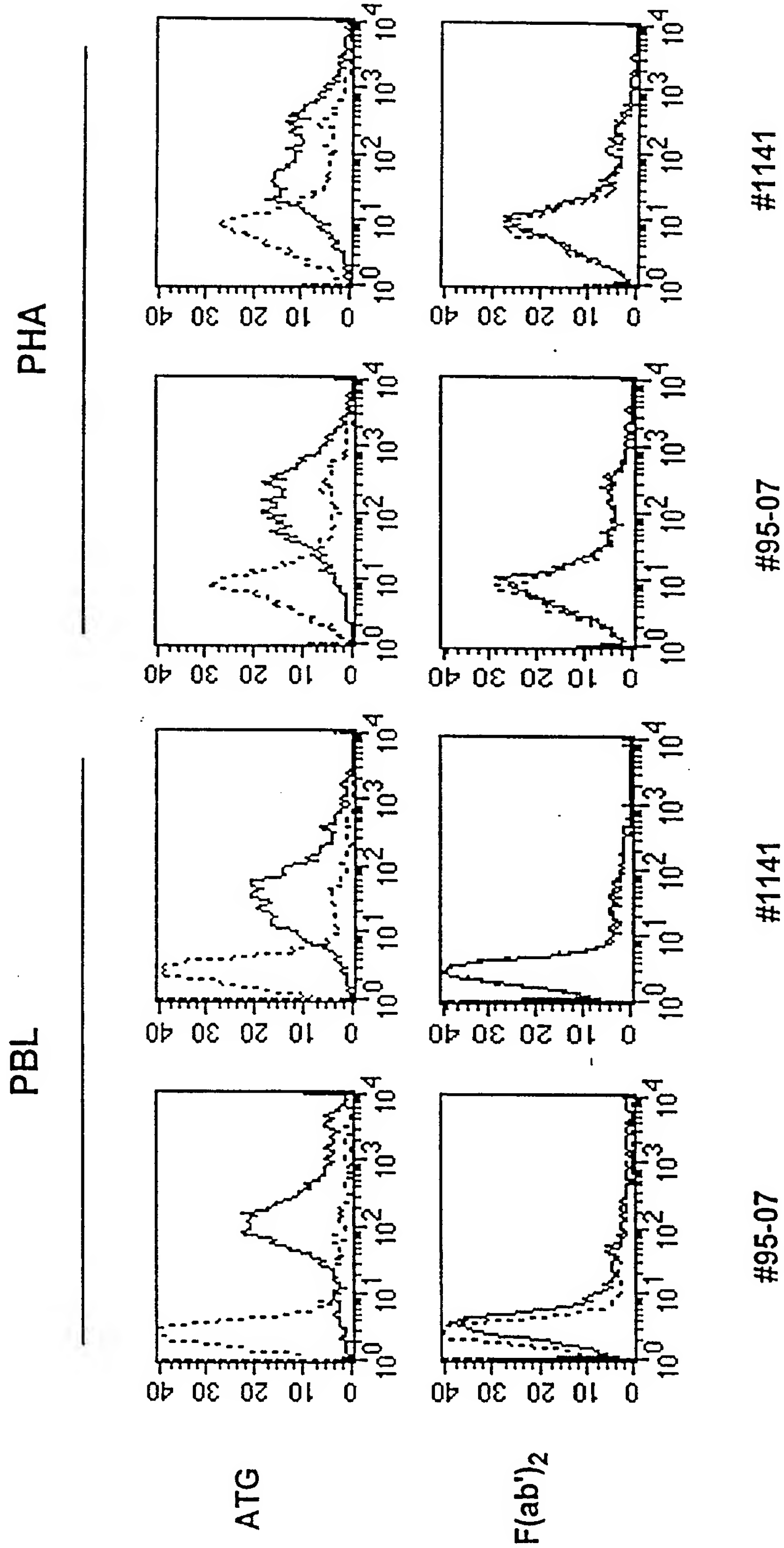

70000
60000
50000
40000
30000
20000
10000
0
CPM
a
a
a
0
50
100
200
Fab Concentrations (ug/ml)
Control Fab
ATG Fab

FIGURE 8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PHA
ATG
F(ab')2
#95-07
#1141
#95-07
#1141
0 10 20 30 40
10^0 10^1 10^2 10^3 10^4

FIGURE 9

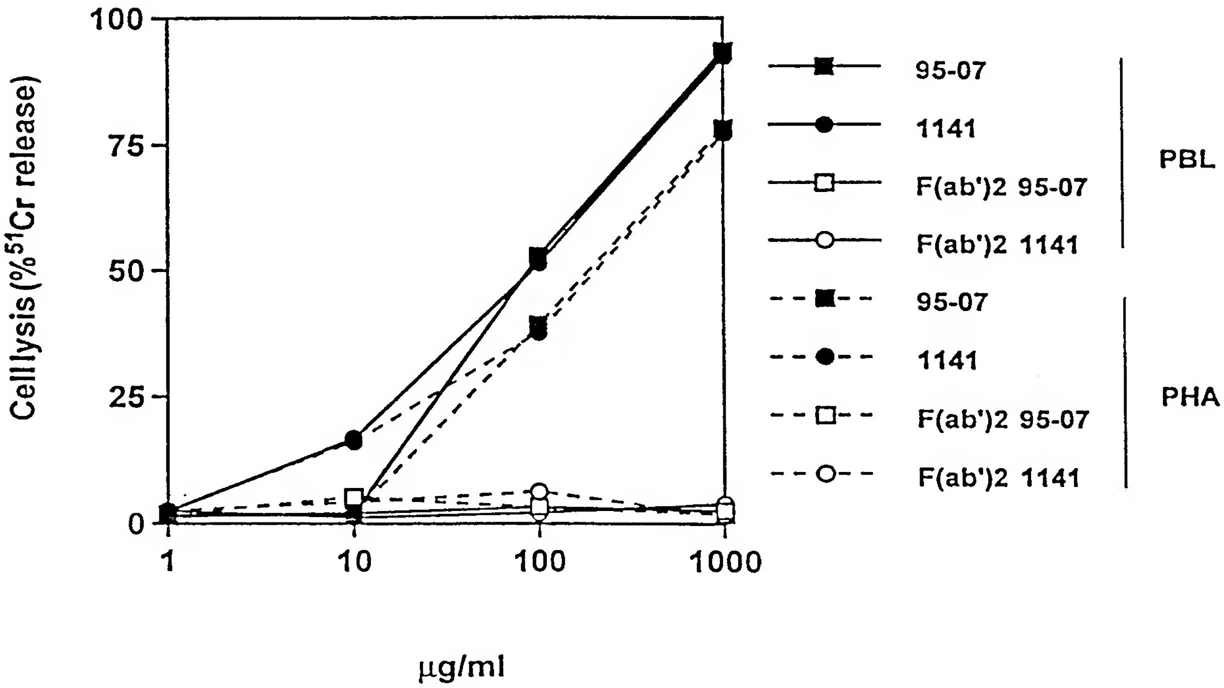
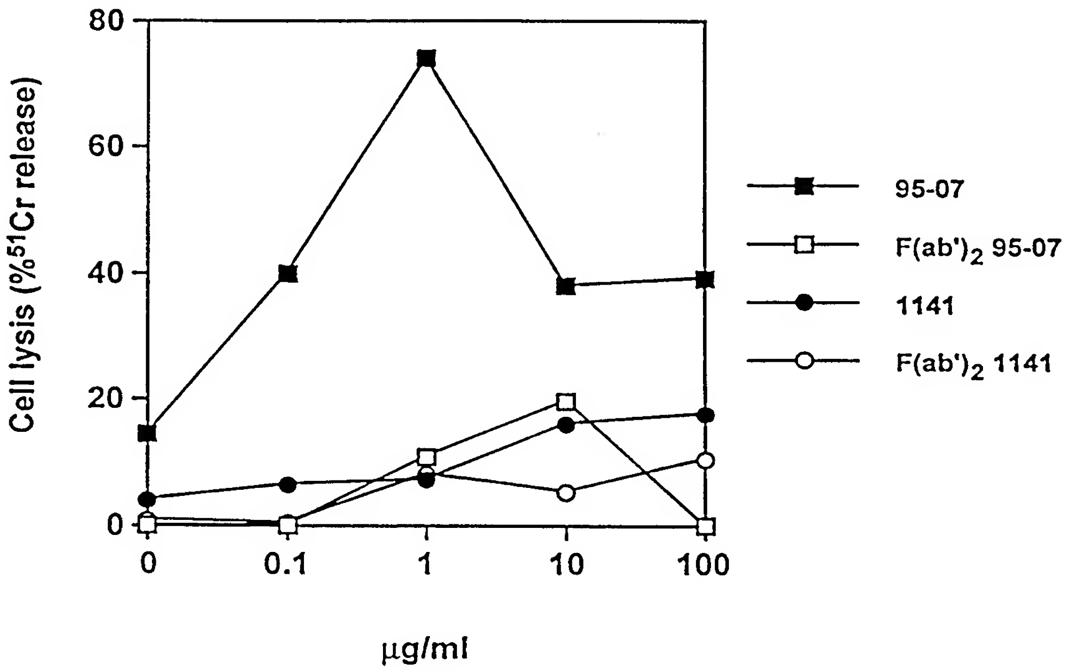

100
75
50
25
0
Celllysis (%51Cr release)
1
10
100
1000
µg/ml
95-07
1141
F(ab')2 95-07
F(ab')2 1141
PBL
95-07
1141
F(ab')2 95-07
F(ab')2 1141
PHA

FIGURE 10



80
60
40
20
0
Cell lysis (%51Cr release)
0
0.1
1
10
100
µg/ml
95-07
F(ab')2 95-07
1141
F(ab')2 1141

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/05829

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) : A61K 39/00, 39/395; C12N 5/02; C07K 16/18, 16/28
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/130.1, 173.1, 177.1, 809, 810; 435/375; 514/ 866, 825, 903, 826, 921; 530/389.1, 389.6, 866, 868

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	WOODRUFF, M.F.A. et al. Effect of antilymphocyte antibody and antibody fragments on human lymphocytes in vitro. Nature. 05 August 1967, Vol. 215, pages 591-594, see entire document.	1-9, 23-28 ---- 10-14, 22
X - Y	GALLAGHER, M.T. et al. Inhibition of the graft-versus-host reaction: I. Reduction of the graft-versus-host potential of mouse spleen cells (with a sparing of stem cells) by treatment with anti-lymphocyte globulin-derived Fab fragments. Transplantation. November 1972, Vol. 14, No. 5, pages 597-602, see entire document.	1, 2, 5-7, 9 ---- 4, 10-18, 21-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 MAY 1999	16 June 1999 (16.06.99)

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	D. Lawrence for F. PIERRE VANDERVEGT
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	BONNEFOY-BERNARD, N. et al. Mechanisms of immunosuppression induced by antithymocyte globulins and OKT3. J. Heart Lung Transplant. May 1996, Vol. 15, pages 435-442, see entire document.	1-8, 10-18, 21 ---- 9, 22
X	BONNEFOY-BERNARD, N. et al. Apoptosis induced by polyclonal antilymphocyte globulins in human B-cell lines. Blood. 15 February 1994, Vol. 83, No. 4, pages 1051-1059, see entire document.	1-6, 8, 23-28
Y	KAVATHAS, P. et al. Transfection for lymphocyte cell surface antigens. In: Handbook of Experimental Immunology. Weir, editor. 1986, Blackwell Scientific Publishers, London, pages 91.1-91.10, see entire chapter.	1, 4, 10, 13
Y	HIRSCH, R. et al. Differential T cell hyporesponsiveness induced by in vivo administration of intact or F(ab')2 fragments of anti-CD-3 monoclonal antibody: F(ab')2 fragments induce a selective T helper dysfunction. J. Immunol. 01 October 1991, Vol. 147, No. 7, pages 2088-2093, see entire document.	1, 7, 10-12, 14-18, 21-22
Y	HIRSCH, R. et al. Effects of in vivo administration of anti-CD3 monoclonal antibody on T cell function in mice: II: In vivo activation of T cells. J. Immunol. 01 February 1989, Vol. 142, No. 3, pages 737-743, see entire document.	1, 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/05829

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/05829

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/130.1, 173.1, 177.1, 809, 810; 435/375; 514/ 866, 825, 903, 826, 921; 530/389.1, 389.6, 866, 868

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

DIALOG medicine index, APS
search terms: polyclonal, antibody, lymphocyte, T cell, complement, Fc receptor, Fab, F(ab')2, activate, stimulate, modulate, supress, downregulate, upregulate

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-22, drawn to drawn to a method for suppressing an immune response.

Group II, claim(s) 1-9 and 23-26, drawn to a method for activating an immune response.

Group III, claim(s) 27-28, drawn to a composition comprising an antilymphocyte antibody.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
The methods of I and II are different methods of use of the product of Group III, i.e., methods of suppressing or stimulating an immune response, respectively, and the methods are therefore diametrically opposed to one another and are not related. The technical feature in common between groups I-III is an antilymphocyte antibodies, or fragments thereof, which modulate an immune response. However, such antibodies and fragments are known in the art as evidenced by Woodruff et al (8/67). Accordingly, the groups are not so linked by a special technical feature as to form a single general inventive concept.